R86Q, a Mutation in *BmAChE3* Yielding a *Rhipicephalus microplus* Organophosphate-Insensitive Acetylcholinesterase

KEVIN B. TEMEYER,[1] JOHN H. PRUETT, PIA UNTALAN OLAFSON, AND ANDREW C. CHEN

Knipling-Bushland U.S. Livestock Insects Research Laboratory, USDA–ARS,
2700 Fredericksburg Road, Kerrville, TX 78028

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ABSTRACT Mutations were identified in the cDNA sequence encoding the acetylcholinesterase BmAChE3 in strains of *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini) resistant or susceptible to organophosphate (OP) acaricide. The mutation that occurred most frequently in the OP-resistant San Román strain resulted in a substitution of glutamine (Q) for arginine (R) at position 86 in BmAChE3 (position 66 in mature BmAChE). Clones containing the mutant and wild-type cDNA sequences were expressed in the baculovirus system. Enzyme kinetics of recombinant BmAChE3 containing or lacking the R86Q mutation demonstrated that the R86Q mutation increased substrate affinity and conferred insensitivity to paraoxon inhibition. This is the first demonstration of a mutation in a gene encoding an ixodid acetylcholinesterase resulting in OP insensitivity. A restriction fragment length polymorphism assay was developed and used to diagnose the frequency of the R86Q mutation in *Bm*AChE3 genomic DNA from seven laboratory-colonized strains. Use of the R86Q diagnostic assay detected an increased frequency of the R86Q mutation in OP-resistant tick strains compared with that of OP-susceptible strains; however, the R86Q mutation was also present in OP-susceptible strains at unexpectedly high frequency. Because the R86Q mutation generates an OP-resistant enzyme in vitro and it is present at an elevated frequency in laboratory strains selected for OP resistance, we conclude that the data are consistent with a potential role for *BmAChE3* in development of OP resistance; however, because the R86Q mutation has a high frequency in susceptible strains, the R86Q mutation alone is insufficient to generate the OP-resistant phenotype at the organismal level. There are likely to be additional mutations in *BmAChE3*, mutations in additional acetylcholinesterase genes, or additional resistance mechanisms (e.g., oxidative metabolism) that contribute to expression of the OP-resistant phenotype.

KEY WORDS OP resistance, *Boophilus*, cattle fever tick, acaricide resistance, Ixodidae

The southern cattle tick, *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini) is an ectoparasite of cattle that vectors the causative agents of bovine babesiosis, *Babesia bovis* and *Babesia bigemina* (Smith and Kilborne 1893), and it was first reported eradicated from the United States in 1943 (Graham and Hourrigan 1977). Because *R. microplus* remains endemic to Mexico, intermittent incursions occur frequently, and reestablishment of *R. microplus* in the United States has been prevented by a continuing surveillance and quarantine program maintained by the Veterinary Services (VS) branch of the Animal and Plant Health Inspection Service of the U.S. Department of Agriculture. All cattle imported into the United States from Mexico are required to be dipped in vats containing the organophosphate (OP) coumaphos (George 1996). There are increasing concerns over reports of OP resistance in Mexico (Santamaria and Fragoso 1994, Fragoso et al. 1995), the recent finding of an OP-resistant outbreak strain in the U.S. quarantine zone (Miller et al. 2005), and the potential failure of the U.S. entry barrier to *R. microplus* (Davey et al. 2003, Temeyer et al. 2004b).

The physiological target site for OP toxicity is the quasi-irreversible inhibition of acetylcholinesterase (AChE, EC 3.1.1.7; O'Brien 1967). AChE insensitivity to OP inhibition in *R. microplus* was first reported by Lee and Bantham (1966), and it was considered the principal resistance mechanism in this species (Bull and Ahrens 1988). In *Drosophila melanogaster* (Meigen) point mutations within the AChE gene result in amino acid substitutions that alter the conformation of AChE, and, as a result, the rate at which it is inhibited by OP (Morton 1993, Fournier and Mutero 1994). Pruett (2002), evaluating the kinetic analysis of OP inhibition of AChE extracted from OP-resistant *R. microplus* strains, observed that the slower rate of OP inhibition of insensitive AChE was most affected by a slower rate of enzyme phosphorylation.

Three cDNAs that encode putative AChEs from *R. microplus* (*BmAChE1*, *BmAChE2*, and *BmAChE3*)

[1] Corresponding author, e-mail: kevin.temeyer@ars.usda.gov.

have been identified, but, to date, no molecular basis for altered AChE activity has been defined, i.e., OP-insensitive AChE (Baxter and Barker 1998, Hernandez et al. 1999, Temeyer et al. 2004a). Currently, biochemical identity of only one of the *BmAChE* cDNAs has been verified with respect to expression and enzymatic properties (Temeyer et al. 2006), and the roles of the individual putative *BmAChEs* remain to be established.

The present work reports identification, expression, biochemical characterization, and population genetics of a mutation in *BmAChE3* cDNA. Results of this study may be of great value in elucidating the genetic mechanism that confers OP resistance in *R. microplus*, and for the first time, demonstrate direct linkage of genetic and biochemical data in a target-insensitive ixodid acetylcholinesterase.

Materials and Methods

Tick Material. *R. microplus* ticks were maintained at the Cattle Fever Tick Research Laboratory (Edinburg, TX). This study used ticks from seven laboratory-reared strains differing in their OP-resistance status (S, susceptible; R, resistant): Gonzalez (S), Muñoz (S), Deutch (S), Santa Luiza (R), Pesqueria (R), San Román (R), and Tuxpan (R). Origin and OP bioassay of the strains are discussed in Li et al. (2003), except for the Deutch and Santa Luiza strains. The Deutch (S) strain was obtained in 2001 from an outbreak in Webb County, TX, ≈20 miles northwest of Laredo, TX. The Santa Luiza (R) strain was derived from amitraz-resistant ticks originating in Brazil provided to us from a colony established at the Centro Nacional de Servicios de Constatacion en Salud Animal, Jiutepec, Morales, Mexico (Li et al. 2004).

Cloning and Sequencing. Total RNA was isolated from *R. microplus* larvae that were ground to a powder by using a liquid nitrogen-cooled mortar and pestle. Total RNA was isolated from the larval powder by using Tri Reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Oligo ($dT_{18}V$) or a gene-specific primer (BmAChE2273L19: 5′-GCTATCATGAGCATGTTTC-3′), designed from the *R. microplus BmAChE3* sequence (Temeyer et al. 2004a; GenBank accession no. AY267337), was used to direct synthesis of first-strand cDNA from the larval RNA template by using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA).

The complete coding region for BmAChE3 was amplified from cDNA of strains Muñoz and San Román by two rounds of high-fidelity polymerase chain reaction (PCR) by using iPROOF DNA polymerase (Bio-Rad, Hercules, CA), according to the manufacturer's instructions, with primer pair BmAChE3-61U15 (5′-CGGTGACCACAGTGC-3′) and BmAChE3-2305L16 (5′-GCTATCATGAGCATGTTTC-3′), followed by nested PCR with primers BmAChE3-150U15 (5′-GGGGAGCACGGTA-3′) and BmAChE3-2246L15 (5′-GCCGTAACAGTGGAA-3′). The amplified cDNA was incubated with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) to add an adenosine at the 3′ terminus, inserted into the pCR4-TOPO vector (Invitrogen), and transformed into chemically competent *Escherichia coli* Stbl 2 (Invitrogen) according to the manufacturer's instructions. Transformants were screened by PCR for the presence of *BmAChE3* cDNA, and for orientation of the insert. Plasmid DNAs were purified from bacterial liquid cultures by using the QIAprep Spin Miniprep kit (QIAGEN, Valencia, CA), and they were sequenced using BigDye terminator (Applied Biosystems) with analysis on an ABI3130XL Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Clones determined to contain the complete, unaltered coding region served as a source of template for high-fidelity PCR amplification to transfer the cDNA to the pBlueBac4.5/V5-HisTOPO vector (Invitrogen) with primer pair BmAChE3-225U19 (5′-CACCATGTACTCGAGGATAGTAG-3′) and BmAChE3-2067L19 (5′-GGGTTTCAGGTAACTTTTC-3′), according to the manufacturer's instructions. Successful constructs (p*BmAChE3*) contained the entire coding sequence of BmAChE3.

Baculovirus Expression. Sf21 insect cells were grown in Grace's insect medium (GIM; Invitrogen) supplemented with 10% fetal calf serum at 27°C according to instructions provided by the vendor. Sf21 cells, at a density of 1.5×10^6 cells ml^{-1}, were cotransfected with p*BmAChE3* and Bac-N-Blue DNA (Invitrogen) and overlaid with agarose containing GIM and X-Gal or Bluo-Gal according to the manufacturer's instructions. Recombinant baculovirus (blue) plaques were picked and used to infect 5-ml cultures of Sf21 cells that were 25% confluent. For baculovirus-infected cultures expressing rBmAChE3 (wild type and R86Q), Sf21 cells were grown in GIM containing fetal calf serum that had been heated for 15–20 min at 65°C (GIMΔ) to inactivate bovine acetylcholinesterase. AChE activity was determined 3–5 d after infection by using a microtiter plate assay (Pruett 2002). The optimum substrate concentration for acetylthiocholine iodide (ASCh; 1.2×10^{-4} M, Sigma-Aldrich, St. Louis, MO) was determined by titration experiments. The substrate was prepared in 50 mM sodium phosphate buffer, pH 7.5 (phosphate buffer), containing 0.32 mM Ellman's reagent, 5,5′-dithio-*bis*(2-nitrobenzoic acid) (Sigma-Aldrich). The standard assay consisted of 20 μl of enzyme and 180 μl of substrate solution. The reaction was monitored with a Dynatech MR5000 plate reader (Dynex Technologies, Chantilly, VA). GIMΔ was used as a negative control for the acetylcholinesterase assay. Absorbance at 405/630 nm was measured at 0, 5, 10, 15, 30, and 60 min after addition of substrate.

Viral stocks were prepared and titer determined by plaque assay as described in the Bac-N-Blue transfection kit instruction manual (Invitrogen). Sf21 cultures were infected with recombinant baculovirus expressing rBmAChE3 (wild type and R86Q) at multiplicities of infection (m.o.i.) of 2, 6, and 12 plaque-forming units $cell^{-1}$. Samples of the infected cultures were withdrawn at 24-h intervals for 3 d and frozen at −70°C until assayed for AChE activity. Because the highest AChE activity was observed between 24 and 48 h

Table 1. *R. microplus* strain San Román mutations altering the amino acid sequence of BmAChE3

Mutation[a]	Nucleotide sequence (5′–3′)[b]	Amino acid sequence[a,b]
I48L	366 **C**TTCCTTTGGAAGCTGGCATA 386	48 **L**PLEAGI 54
Wild type	A.....	I.....
I54V	366 ATTCCTTTGGAAGCTGGC**G**TA 386	48 IPLEAG**V** 54
Wild type	.....A..	I
R86Q	475 CTTCAC**A**GCCTATATCGACAG 495	84 TS**Q**PIST 90
Wild type	.....G.....	..R....
V137I	624 CGCAATCCC**A**TCCCTGTGAAG 644	134 RNP**I**PVK 140
Wild type	.....G.....	...V...
I492M	1692 TTCGCCAT**G**ATTTGCCCGACC 1712	490 FA**M**ICPT 496
Wild type	.....C.....	..I....
T548A	1863 CCT**G**CGATTGCCACCGACCAG 1883	547 P**A**IATDQE 554
Wild type	...A.....	.T.....

[a] Numbering of amino acids is from the initiation codon of BmAChE3.
[b] Substitutions are indicated below boldface wild-type sequence.

postinfection in cultures of high m.o.i., 50 ml of Sf21 shake flask cultures were infected at an m.o.i. of 10 and incubated at 27°C. Thirty hours after infection, culture supernatants were collected by centrifugation, assayed for AChE activity, and used to determine biochemical kinetics of rBmAChE3 (wild type and R86Q).

Determination of K_m and V_{max} for rBmAChE3 (Wild Type and R86Q). The general assay for AChE activity was conducted in microplates with ASCh and butyrylthiocholine iodide as substrates by using the method described above. The standard assay consisted of 20 μl of enzyme and 180 μl of substrate solution. The reaction was monitored at 405 nm with a Bio-Tek EL808 Ultra Microplate Reader (Bio-Tek Instruments, Winooski, VT) for 1 h, with readings every 10 min.

To determine the K_m and V_{max} values for rBmAChE3 (wild type and R86Q), substrate concentrations ranged from 480 to 30 μM (serial dilutions). K_m and V_{max} values were calculated with the aid of SigmaPlot Enzyme Kinetics Module 1.2 (Systat Software Inc., Point Richmond, CA) on the resultant initial velocities (V_o).

Determination of Bimolecular Rate Constants for rBmAChE3 (Wild Type and R86Q) by Inhibition with Paraoxon. Each enzyme was concentrated ≈3.5 times, and the rate of rBmAChE3 (wild type and R86Q) inhibition was determined in the presence of 0.12 mM ASCh by using six concentrations of paraoxon (3.5, 3.0, 2.5, 2.0, 1.5, and 1.0×10^{-6} M). The progressive inhibition of each enzyme was monitored over time (12 min, readings at 2-min intervals). The natural log of the percentage of residual rBmAChE3 (wild type and R86Q) activity at each reading for each paraoxon concentration was plotted against time. The apparent rate constant (k), the slope of the line for each paraoxon concentration, was determined by linear regression of the data points. The values for k_i, k_2, and K_d were determined by a double reciprocal plot of the apparent rate constants ($1/k$) against the inhibitor concentrations ($1/[I]\ (1 - \alpha)$). The value of α was calculated as $[S]/(K_m + [S])$ (Chen et al. 2001).

Diagnostic PCR-Restriction Fragment Length Polymorphism Assay for BmAChE3-R86Q Mutation. Genomic DNA samples were prepared from individual tick larvae by grinding in 100 μl of TEN buffer (10 mM Tris-HCl, pH 8.0, 25 mM NaCl, and 0.1 mM Na_2EDTA). Samples were heated 10 min at 99.5°C and centrifuged for 2 min at 14,000 × *g*. A fragment of *BmAChE3* DNA containing the R86Q site was amplified by PCR with primers Ace3R86Q-277U20 (5′-CTCATGCGAGATGCTACATA-3′) and Ace3R86Q-568L17 (5′-GCCGACGAATTGGTAGA-3′) flanking R86Q. One microliter of genomic DNA preparation was added as template to 15 μl of PCR mastermix (0.5 μM each primer Ace3R86Q-277U10 and Ace3R86Q-568L17, 50 μM each dNTP, and 0.005 U/μl JumpStart REDAccuTaq LA DNA polymerase; Sigma-Aldrich) and incubated 30 s at 96°C followed by 35 cycles, each consisting of 30 s at 94°C, 1 min at 61°C, and 2 min at 68°C. PCR cycling was followed by a final incubation for 5 min at 68°C. Five microliters of the PCR mix containing the 308-bp amplification product was incubated for 2 h at 37°C with HaeIII restriction endonuclease (New England BioLabs, Ipswich, MA) in the supplier-recommended buffer. Products of the restriction digestion were analyzed by electrophoresis on a 3.5% Metaphor agarose (Cambrex Bio Science, Rockland, ME) gel by using Hi-Lo DNA Marker (Minnesota Molecular Inc., Minneapolis, MN) as standards.

Results

BmAChE3 cDNA Sequence in OP-Resistant and Susceptible Strains. Complete coding sequences were obtained for seven clones of *BmAChE3* cDNA from an OP-resistant (San Román) and an OP-susceptible (Muñoz) strain of *R. microplus.* Mutations resulting in alteration to the amino acid sequence of BmAChE3 in the San Román strain are summarized in Table 1 (amino acid numbering includes the leader peptide). These mutations were frequently identified in combination; for example, one isolate contained five of the six mutations listed in Table 1, whereas other isolates contained three, two, or one. It was noted that the R86Q mutation (position 66 in the mature protein), the only mutation found in the absence of other mutations (data not shown), was the alteration most frequently observed in the San Román strain (OP-resistant), a strain that had previously been shown to exhibit a decreased K_m (increased affinity for sub-

Table 2. Biochemical characterization of rBmAChE3 (wt) and rBmAChE3-R86Q (mutant) by calculation of K_m and V_{max} with substrate ASCh and determination of bimolecular rate constant k_i, phosphorylation constant k_2, and dissociation constant K_d, in the presence of paraoxon

Expression construct	V_{max}[a] (μ mol min^{-1})	K_m[a] (μ mol)	k_i ($\times 10^4$ mol min^{-1})	k_2 (min)	K_d ($\times 10^{-6}$ M)
r*Bm*AChE3	2.20 ± 0.024	124.80 ± 3.58	5.11	1.60	31.33
r*Bm*AChE3-R86Q	0.36 ± 0.015	99.60 ± 11.68	2.97	0.08	2.80

[a] ± SE.

strate) for total acetylcholinesterase activity in extracts of whole larvae (Li et al. 2005) and adult synganglia (Pruett 2002).

Biochemical Characterization of rBmAChE3 Containing the R86Q Substitution. Baculovirus expression clones, isogenic except at the R86Q locus of *BmAChE3* cDNA, were constructed and transfected into Sf21 insect cells for expression. Recombinant BmAChE3 expressed in culture supernatant was used for biochemical characterization and enzyme kinetics. Results of biochemical characterization in terms of V_{max}, K_m, and bimolecular rate constants in the presence of paraoxon, are presented in Table 2. Although differences in V_{max} observed between the wild-type and R86Q mutant constructs may reflect different enzyme concentrations in different preparations, differences in K_m values indicate an increased affinity for substrate (ASCh) in the R86Q mutant. A slower rate of enzyme inhibition by paraoxon in the mutant is reflected in the lower k_i and k_2 values. The wild-type enzyme is phosphorylated 19.2-times faster than the mutant.

Development of Diagnostic PCR-Restriction Fragment Length Polymorphism-Based Assay for the R86Q Mutation and Survey of Laboratory Strains. As previously noted in Table 1, the R86Q amino acid sequence substitution is the result of a single nucleotide change of A replacing G at position 481. This nucleotide substitution abolishes the HaeIII restriction endonuclease site, enabling use of HaeIII to diagnose the presence or absence of the R86Q mutation. Oligodeoxynucleotide primers were designed to specifically amplify a 308-bp genomic DNA fragment containing the R86Q locus, the product of which was subsequently digested with HaeIII and analyzed electrophoretically. In silico HaeIII digestion of the wild-type sequence (86-R) predicted bands at 206, 56, and 46 bp, whereas the R86Q mutant sequence is cleaved to yield bands at 262 and 46 bp. Therefore, the diagnostic bands are the 262-bp band that indicated presence of the R86Q sequence and the 206-bp band that indicated presence of the wild-type sequence. The assay was validated using *BmAChE3* clones differing only at the R86Q locus. Although additional HaeIII sites were located within the amplified fragment, they did not interfere with diagnostic interpretation of the digest (Fig. 1).

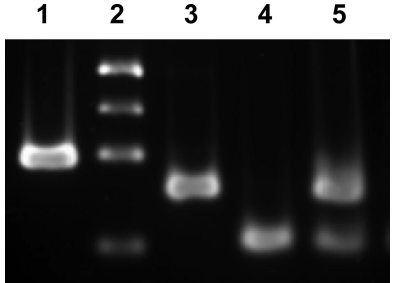

Fig. 1. PCR-restriction fragment length polymorphism assay for R86Q–HaeIII digestion of the 308-bp PCR product containing the R86Q locus. Lane 1, undigested 308-bp PCR product; lane 2, Hi-Lo DNA standards (bands at 200, 300, 400, and 500 bp); lane 3, homozygous R86Q mutant (262 bp); lane 4, homozygous wt (206 bp); and lane 5, heterozygous for R86Q locus (262 + 206 bp).

Genomic DNA was isolated from ≈25 individual larvae per strain for seven laboratory-colonized strains of *R. microplus.* Presence of the R86Q allele was evaluated for individual larvae by using the diagnostic assay. Frequency of R86Q genotypes for each of the strains is summarized in Table 3. The Tuxpan strain (R) seemed to be 100% homozygous for the R86Q mutation, whereas the other strains had various frequencies.

Discussion

Three cDNAs putatively encoding AChEs (*BmAChE1*, *BmAChE2*, and *BmAChE3*) were previously reported for *R. microplus;* however, no mutations have been reported within the three *BmAChEs* that correlated with resistance to OPs (Baxter and Barker 1998, Hernandez et al. 1999, Temeyer et al. 2004a). The inability to identify mutations associated with resistance within *BmAChE1* and *BmAChE2* prompted the suggestion

Table 3. Frequency of *BmAChE3-R86Q* genotypes in strains of *R. microplus* assessed by PCR-restriction fragment length polymorphism diagnostic assay

Strain	n	RR[a]	RQ[a]	QQ[a]	F_Q[b]	rr[c]	rr[d]
Deutch	24	6	13	5	0.48		
Gonzalez	25	2	17	6	0.58	1.00	1.00
Muñoz	25	3	13	9	0.62	1.07	0.64
San Román	22	1	11	10	0.70	10.09	9.5
Pesqueria	24	2	9	13	0.73	3.57	
Santa Luiza	24	0	2	22	0.96	5.50[e]	2.62[e]
Tuxpan	25			25	1.00	5.86	18.29

[a] RR, homozygous wild-type; RQ, heterozygous; and QQ, homozygous mutant.
[b] Frequency (86Q).
[c] Resistance ratio (coumaphos; Li et al. 2003).
[d] Resistance ratio (diazinon; Li et al. 2003).
[e] Resistance ratio (Li et al. 2004).

that resistance might result from posttranslational modification of the acetylcholinesterase (Baxter and Barker 1998, 2002). Here, we report multiple mutations in *BmAChE3* of the OP-resistant San Román strain of *R. microplus* (Table 1).

Several expression constructs containing different individual mutations listed in Table 1 were evaluated in our expression system and found to result in low acetylcholinesterase activity, suggesting that these mutations resulted in production of an enzyme with significantly reduced activity, or reduced stability. The low activity of these constructs relative to background prevented biochemical characterization. However, the construct containing R86Q yielded sufficient activity to enable biochemical characterization. This mutation mapped to the outer shell well away from the catalytic triad or active site gorge on a molecular model (Chen et al. 2003) based on the backbones of acetylcholinesterases from *D. melanogaster* (*1QO9_A*) and *Torpedo californica* (*1EA5*). Previous studies of natural acetylcholinesterase extracted from larvae of the OP-resistant *R. microplus* San Román strain demonstrated a lower level of AChE activity (reduced V_{max} and K_m) and a slower rate of phosphorylation that were equivalent for both paraoxon and coroxon, relative to a control susceptible strain (Pruett 2002, Li et al. 2005, Pruett and Pound 2006). Here, we demonstrate that presence of the R86Q mutation found in a recently identified acetylcholinesterase gene of *R. microplus* (*BmAChE3;* Temeyer et al. 2004a, 2006) results in an altered enzyme exhibiting both insensitivity to paraoxon inhibition and similar kinetic characteristics to the natural acetylcholinesterase found in larval extracts.

Because the R86Q mutation confers characteristics of an OP-insensitive acetylcholinesterase, a diagnostic assay was developed to evaluate the frequency of the mutation and its correlation with resistance. The highest frequency of the resistant allele (86Q) was found in the Tuxpan (100%), Santa Luiza (96%), Pesqueria (73%), and San Román (70%) strains, all of which are characterized as OP-resistant (Li et al. 2003). Similarly, Pruett and Pound (2006), using enzyme inhibition kinetics to differentiate OP-resistant genotypes, reported resistant allele frequencies of 75 and 65% for Pesqueria and San Román, respectively. These results are consistent with the hypothesis of an association between R86Q and OP resistance, possibly highlighting a role for the *BmAChE3* gene in the OP-resistance mechanism. However, in the susceptible strains (Gonzalez, Muñoz, and Deutch), the R86Q allele frequency, although lower relative to the resistant strains, was still present at a frequency of 48 to 62% in contrast to an 11% frequency of the resistance allele in the Muñoz strain reported by Pruett and Pound (2006). It is important to note that the resistance allele referred to by Pruett and Pound (2006) is a biochemical phenotype of unknown origin. The presence of the R86Q mutation in OP-susceptible strains of *R. microplus* strongly suggests that although the R86Q mutation may contribute to resistance development, it alone is insufficient to produce the resistant phenotype. Our kinetic studies of the recombinant BmAChE3 demonstrate that the R86Q mutation does result in relative insensitivity of the BmAChE3 enzyme to OP-inhibition, reflecting a slower rate of enzyme phosphorylation, as was previously noted by Pruett (2002) for total AChE activity extracted from OP-resistant *R. microplus* strains. We interpret the high frequency of the R86Q mutation in resistant strains and its biochemical effects in vitro as suggestive of the direct involvement of *BmAChE3* in the resistance mechanism, but it does not exclude the involvement of other mutations or other factors. Li et al. (2005) reported that the mechanism of coumaphos resistance in the San Román strain of *R. microplus* involves both insensitive AChE and an enhanced cytochrome P450-based metabolic detoxification. It has been well established that multiple mutations in an AChE may be additive, generating increased resistance to OP inhibition (Villatte et al. 2000, Chen et al. 2001, Fournier 2005); therefore, phenotypic OP resistance may involve multiple mutations in the same gene, or it may even be multigenic. The OP-resistant phenotype is characterized by tick survival in the presence of OP. If *R. microplus* has multiple *BmAChE* genes, it is reasonable to expect that they each may play a vital role in organismal biology and that mutation in more than one of the *BmAChE*s may be essential to survival in bioassays, which would explain the initial failure to find mutations in *BmAChE1*, *BmAChE2*, or *BmAChE3* correlating with phenotypic resistance. Further investigation of the biochemical effects of additional mutations in *BmAChE3* of *R. microplus* strain San Román (Table 1) that have yet to be characterized may demonstrate a role in production of an enzyme with increased OP insensitivity (Villatte et al. 2000). In addition, the respective roles of the multiple acetylcholinesterases of *R. microplus* remain to be elucidated.

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References Cited

Baxter, G. D., and S. C. Barker. 1998. Acetylcholinesterase cDNA of the cattle tick, *Boophilus microplus:* characterization and role in organophosphate resistance. Insect Biochem. Mol. Biol. 28: 581–589.

Baxter, G. D., and S. C. Barker. 2002. Analysis of the sequence and expression of a second putative acetylcholinesterase cDNA from organophosphate-susceptible and organophosphate-resistant cattle ticks. Insect Biochem. Mol. Biol. 32: 815–820.

Bull, D. L., and E. H. Ahrens. 1988. Metabolism of coumaphos in susceptible and resistant strains of *Boophilus microplus* (Acari: Ixodidae). J. Med. Entomol. 25: 94–98.

Chen, J., J. B. Anderson, C. DeWeese-Scott, N. D. Fedorova, L. Y. Geer, S. He, D. I. Hurwitz, J. D. Jackson, A. R. Jacobs,

C. J. Lanczycki, et al. 2003. MMDB: Entrez's 3D-structure database. Nucleic Acids Res. 31: 474–477.

Chen, Z., R. Newcomb, E. Forbes, J. McKenzie, and P. Batterham. 2001. The acetylcholinesterase gene and organophosphorous resistance in the Australian sheep blowfly, *Lucilia cuprina.* Insect Biochem. Mol. Biol. 31: 805–816.

Davey, R. B., J. E. George, and R. J. Miller. 2003. Efficacy of various concentrations of coumaphos to control adult, nymphal, and larval stages of an organophosphate-resistant strain of *Boophilus microplus* on infested cattle. Am. J. Vet. Res. 64: 684–689.

Fournier, D. 2005. Mutations of acetylcholinesterase which confer insecticide resistance in insect populations. Chem. Biol. Interact. 157–158, 257–261.

Fournier, D., and A. Mutero. 1994. Modification of acetylcholinesterase as a mechanism of resistance to insecticides. Comp. Biochem. Physiol. 108C: 19–31.

Fragoso, H., N. Soberanes, M. Ortiz, M. Santamaria, and A. Ortiz. 1995. Epidemiology of ixodicide resistance in the *Boophilus microplus* ticks in Mexico, pp. 45–47. *In* Third International Seminar of Animal Parasitology, 11–13 October 1995, SAGAR-CANIFARMA-FAO-IICA-INIFAP, Acapulco, Mexico. Calz. de la Cerillera, Jiutepec, Mor. Mexico.

George, J. E. 1996. The campaign to keep *Boophilus* ticks out of the United States: technical problems and solutions, pp. 196–206. *In* G. G. Wagner and W. W. Buisch [eds.], Proceedings of the 100th Annual Meeting of the U.S. Animal Health Assoc, 12–18 October, Little Rock, AR. Spectrum Press, Richmond, VA.

Graham, O. H., and J. L. Hourrigan. 1977. Eradication programs for the arthropod parasites of livestock. J. Med. Entomol. 13: 629–658.

Hernandez, R., H. He, A. C. Chen, G. W. Ivy, J. E. George, and G. G. Wagner. 1999. Cloning and sequencing of a putative acetylcholinesterase from *Boophilus microplus* (Acari: Ixodidae). J. Med. Entomol. 36: 764–770.

Lee, R. M., and P. Bantham. 1966. The activity and organophosphate inhibition of cholinesterase from susceptible and resistant ticks (Acari). Entomol. Exp. Appl. 9: 13–24.

Li, A. Y., R. B. Davey, R. J. Miller, and J. E. George. 2003. Resistance to coumaphos and diazinon in *Boophilus microplus* (Acari: Ixodidae) and evidence for the involvement of an oxidative detoxification mechanism. J. Med. Entomol. 40: 482–490.

Li, A. Y., R. B. Davey, R. J. Miller, and J. E. George. 2004. Detection and characterization of amitraz resistance in the southern cattle tick, *Boophilus microplus* (Acari: Ixodidae). J. Med. Entomol. 41: 193–200.

Li, A. Y., J. H. Pruett, R. B. Davey, and J. E. George. 2005. Toxicological and biochemical characterization of coumaphos resistance in the San Roman strain of *Boophilus microplus* (Acari: Ixodidae). Pestic. Biochem. Physiol. 81: 145–153.

Miller, R., R. B. Davey, and J. E. George. 2005. First report of organophosphate-resistant *Boophilus microplus* (Acari: Ixodidae) within the United States. J. Med. Entomol. 45: 912–917.

Morton, R. A. 1993. Evolution of *Drosophila* insecticide resistance. Genome 36: 1–7.

O'Brien, R. D. 1967. Insecticides action and metabolism. Academic, New York.

Pruett, J. H. 2002. Comparative inhibition kinetics for acetylcholinesterases extracted from organophosphate resistant and susceptible strains of *Boophilus microplus* (Acari: Ixodidae). J. Econ. Entomol. 95: 1239–1244.

Pruett, J. H., Jr., and J. M. Pound. 2006. Biochemical diagnosis of organophosphate-insensitivity with neural acetylcholinesterase extracted by sonication from the adult tick synganglion. Vet. Parasitol. 135: 355–363.

Santamaria, E. M., and S. H. Fragoso. 1994. Resistencia en garrapatas *Boophilus microplus,* a los ixodicides en Mexico, pp. 473–474. *In* J. M. Perez Trujillo and E. Gonzales Padila [eds.], Proceedings, 14th Pan American Congress on Veterinary Sciences, 9–15 October, Acapulco, Mexico. Casa Autrey, D. F., Mexico.

Smith, T., and F. L. Kilborne. 1893. Investigation into the nature, causation, and prevention of Texas or Southern cattle fever. U.S. Dep. Agric. Bull. 1, BAI.

Temeyer, K. B., R. B. Davey, and A. C. Chen. 2004a. Identification of a third *Boophilus microplus* (Acari: Ixodidae) cDNA presumptively encoding an acetylcholinesterase. J. Med. Entomol. 41: 259–268.

Temeyer, K. B., J. M. Pound, J. A. Miller, A. C. Chen, J. H. Pruett, F. D. Guerrero, R. B. Davey, P. M. Untalan, K. H. Lohmeyer, A. Y. Li, R. J. Miller, and J. E. George. 2004b. Organophosphate resistance in Mexican strains of *Boophilus microplus:* a major threat to the U.S. cattle industry. SAAS Bull. Biochem. Biotechnol. 17: 43–51.

Temeyer, K. B., J. H. Pruett, P. M. Untalan, and A. C. Chen. 2006. Baculovirus expression of *Bm*AChE3, a cDNA encoding an acetylcholinesterase of *Boophilus microplus* (Acari: Ixodidae). J. Med. Entomol. 43: 707–712.

Villatte, F., P. Ziliani, V. Marcel, P. Menozzi, and D. Fournier. 2000. A high number of mutations may provide insecticide-resistance in insect acetylcholinesterase. Pestic. Biochem. Physiol. 67: 95–102.

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